IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Roy Larsen et al.	Confirmation No.:	2306
Serial No.:	10/766,057	Art Unit:	1618
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Title:	RECEPTOR BINDING CONJUGATES		

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APPELLANT'S BRIEF ON APPEAL PURSUANT TO 37 C.F.R. § 41.37

In support of Appellant's Notice of Appeal that was filed in connection with the above-captioned case on February 4, 2011, and with reference to the final Office Action that was mailed in this case on August 4, 2010[1], submitted herewith is Appellant's Appeal Brief.

[1] Appellants note that, while the Office Action Summary lists the action as "non-final," the Office Action, at page 7, indicates that the action has been made final. In any event, the pending claims have been twice rejected.

TABLE OF CONTENTS

Real Party in Interest

The real party in interest in this case is Algeta AS, to whom all interest in the present application has been assigned.

Related Appeals and Interferences

There are no pending appeals or interferences related to this case.

Status of Claims

Claims 18 and 25-35 are pending. Claims 1-17 and 19-24 have been cancelled. Claims 18 and 25-35 were finally rejected in the final Office Action issued on August 4, 2010 and are appealed.

Status of Amendments

All amendments to date have been entered.

Summary of Claimed Subject Matter

As outlined at page 2, lines 12-16, of the specification Appellants' invention features dual binding conjugates containing a radionuclide, an antibody, antibody fragment, or antibody construct having affinity for a tumor associated antigen, and a non-cytotoxic folate. The combination of folate targeting and antibody targeting allows for better targeting of the cytotoxic radionuclide to tumor cells because, as explained at page 3, lines 27-32 of the specification, expression of folate binding protein and the antigen may vary in the target cells and subpopulations and, therefore, the dual binding conjugates allow for an increased probability of achieving therapeutically sufficient targeting of all of the tumor cells. A concise explanation of the subject matter defined in each independent claim is provided below.

Claim 18

Claim 18 is directed to a method or targeting a radionuclide to a malignant cell within a subject, where the malignant cell expresses a tumor associated antigen and expresses folate binding protein (described, for example, at page 3, lines 9-36, of the specification). The method involves (i) coupling an antibody, antibody fragment, or antibody construct having affinity for the tumor antigen to at least one non-cytotoxic folate to form a dual binding conjugate (described, for instance, in Example 1 at pages 7 and 8 of the specification), (ii) coupling the radionuclide to the dual binding conjugate (described, for instance in Example 2, at pages 8 and 9 of the specification), and (iii) administering the radionuclide coupled to the dual binding conjugate to the subject (described, for instance, in Example 3, at pages 9 and 10 of the specification).

Claim 31

Claim 31 is directed to a conjugate consisting of (i) a radionuclide, (ii) an antibody, antibody fragment, or antibody construct, with affinity for a tumor antigen, and (iii) at least one non-cytotoxic folate (described, for instance, at page 2, lines 20-27, and in Example 6 of the specification).

Grounds of Rejection to be Reviewed on Appeal

The ground of rejection on appeal is whether the Office erred in rejecting claims 18 and 25-35 under 35 U.S.C. § 103(a) as being unpatentable over Wedeking et al. (U.S. Patent No. 6,093,383) in view of Sinkule et al. (European Patent Application No. 282057).

Argument

In the final Office Action issued on August 4, 2010, the Office maintained the rejection of claims 18 and 25-35 under 35 U.S.C. § 103(a) as being unpatentable over Wedeking et al. (U.S. Patent No. 6,093,383; hereafter "Wedeking") in view of Sinkule et

al. (European Patent Application No. 282057; hereafter "Sinkule"). Appellants, for the reasons explained below, submit that neither claim 18 nor claim 31, which are directed to Appellants' discovery that both an antibody component and a non-cytotoxic folate component of a conjugate can be used to target a radionuclide to a malignant cell, is rendered obvious by the combination of Wedeking and Sinkule.

Claim 18

As summarized above, claim 18 is directed to a method of targeting a radionuclide to a malignant cell within a subject, where the malignant cell expresses a tumor associated antigen and expresses folate binding protein. This method involves (i) coupling an antibody, antibody fragment, or antibody construct having affinity for the tumor associated antigen to at least one non-cytotoxic folate to form a dual binding conjugate, (ii) coupling the radionuclide to the dual binding conjugate, and (iii) administering the radionuclide coupled to the dual binding conjugate to the subject. Appellants submit that nothing in the cited art renders obvious the dual binding nature of the complexes encompassed by the claims.

In response to Appellants' argument that Sinkule fails to describe targeting the conjugate to a malignant cell using the folate component of a complex, and that, instead, Sinkule explicitly states that the antibody component targets the complex to the tumor, the Office states (page 5 of the August 4, 2010 Office Action):

> The antibody targeting/binding of the radionuclide-folate analogue complex of Sinkule does not exclude simultaneous and/or synergistic folate targeting/binding. The IgG of Sinkule et al. encompasses the IgG antibody of the instant claims and therefore is capable of the same functions, such as not interfering with the targeting of folate and has the same properties.

Appellants respectfully disagree.

While the conjugate of Sinkule may contain folic acid analogues, these folic acid analogues are described as *chemotherapeutic* agents (see column 2, lines 26-30). As such, the folic acid analogue forms part of the "*therapeutic activity*" of the conjugate that

is localized by the antibody. Sinkule states, at column 5, lines 13-15, "[t]he therapeutic activity of the conjugate in vivo is localized by the antibody, which is selected for specificity for the target cell or biomaterial." Sinkule does not teach or suggest folate targeting.

Given that, in Sinkule, the *antibody* is targeting the complex, one simply cannot conclude that the presence of the antibody does not interfere with folate targeting. On this point, Appellants directed the Office's attention to knowledge in the art showing that adding a large molecule, such as bovine serum albumin (BSA), to a folate-containing complex can interfere with the targeting ability of the folate. The Office dismisses Appellants' argument stating (page 6 of the final Office Action):

> BSA is not structurally or chemically identical to IgG and thus does not exactly replicate IgG's actions/interactions in vivo.

Appellants submit that the effect of BSA (a large protein with a mass of about 66,000 amu) on folate targeting is relevant to what one of skill the in art would expect to observe if another large protein (e.g., an IgG antibody, which has a mass of about 150,000 amu) is coupled to a folate-targeted complex. With respect to BSA, the specification states (at page 1, lines 22-27; a copy of Shinoda is provided in the evidence appendix):

> In a previous study, Shinoda et al. (1998) evaluated folate conjugated bovine serum albumin (BSA) labelled with the radionuclide indium-111, and found that there was a significant difference in pharmacokinetics and biodistribution of non-folate compared to folate labelled BSA. A high liver uptake and rapid blood clearance indicated that the folate labelled version of ^{111}In-BSA was not particularly suitable for radionuclide delivery to tumour cells expressing folate binding protein.

The folate in Shinoda's BSA-folate-radionuclide complex cannot be considered to target the radionuclide to the tumor because, once BSA is added to the complex, presence of the folate is not sufficient for the complex to be localized to the tumor. In contrast, Appellants' claims are directed to conjugates in which a non-cytotoxic folate maintains its targeting ability when complexed with an antibody and a radionuclide; the claims recite

dual binding conjugates containing an antibody component that targets a tumor associated antigen and a non-cytotoxic folate component that targets folate binding protein. Appellants submit that, in view of Shinoda, one skilled in the art would not have considered that a large molecule, like an antibody, could be complexed with a folate with the expectation that the folate would maintain its targeting ability. Nothing in the art relied on by the Office in making the present obviousness rejection provides any teaching or suggestion to the contrary.

The Office maintains that the "IgG of Sinkule et al. encompasses the IgG antibody of the instant claims and therefore is capable of the same functions, such as not interfering with the targeting of folate and has the same properties" (August 4, 2010 Office Action at page 6). Appellants respectfully submit that the Office's reliance on inherency is misplaced.

The M.P.E.P. states (section 2112(IV); emphasis original):

> The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (reversed rejection because inherency was based on what would result due to optimization of conditions, not what was necessarily present in the prior art); *In re Oelrich*, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.' " *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) (citations omitted) (The claims were drawn to a disposable diaper having three fastening elements. The reference disclosed two fastening elements that could perform the same function as the three fastening elements in the claims. The court construed the claims to require three separate elements and held that the reference did not disclose a separate third fastening element, either expressly or inherently.).

For example, a rejection for obviousness relying on the undisclosed but inherent heat transfer properties of foam was reversed by the U.S. Court of Customs and Patent Appeals in *Application of Harold W Adams,* 356 F.2d 998 (C.C.P.A. 1966). The court stated:

> [f]inally, the solicitor adds the argument that the *superiority* of appellant's heat transfer is *inherent* in the use of foam. Again we observe that, of course, it is. But the art does not suggest the use of foam in heat transfer of any kind and there is not the slightest suggestion that anyone *knew* of the existence of this inherent superiority until Adams disclosed it. After all, Bell's telephone was "inherently" capable of transmitting speech, DeForest's triode was "inherently" capable of amplification, and, to come down to date, so was the tiny transistor which is rapidly supplanting it. Two of our decisions are cited as supporting the erroneous notion that "subject matter cannot be patented on the basis of an inherent property." We think the proposition thus broadly stated and as applied here is so transparently erroneous as not to require discussion. (Emphasis added).

Appellants, surprisingly, have shown that <u>both</u> the antibody component and the non-cytotoxic folate component of a conjugate can target the conjugate to a malignant cell (i.e., the folate maintains its targeting ability even when complexed with a large protein). The issue is not whether the antibody component is inherently capable of not "interfering with the targeting of folate." The ability of folate to maintain its targeting ability is surprising in view of state of the art as evidenced by Shinoda. In this regard, Appellants direct the Office's attention to Examples 5 and 6 of the specification. In particular, in Example 6, at page 14, lines 18-23, the specification states:

> Folate-antibody-radionuclide conjugates show a significant binding to folate binding protein (FBP) on cells indicating that these conjugates may be used to target FBP-expressing tumour cells in vivo. Also, as demonstrated by specific binding of folate-TP-3-IgG-^{125}I to antigen positive OHS cells as well as FBP-positive HELA-S3 and OVCAR-3 cells, folate-antibody-radionuclide conjugates can possess dual binding ability.

Appellants, for the reasons explained above, submit that Sinkule does <u>not</u> describe <u>folate targeting</u> or use of folate to target a conjugate. The antibody targets the Sinkule

conjugate. While the antibody component of the conjugates encompassed by the present claims may be analogous to the IgG antibodies described by Sinkule, nothing in the Sinkule teaches or suggests that, in a complex containing both an antibody component and a folate component, the folate is capable of targeting the complex.

The Office, nonetheless, maintains that "the antibody targeting/binding of the radionuclide-folate analogue complex of Sinkule does not exclude simultaneous and/or synergistic folate targeting/binding" (Office Action at page 6). While Sinkule does not exclude folate binding, as stated above, in view of Shinoda, one skilled in the art would have expected that inclusion of a large protein, such as an antibody, in a radionuclide/folate complex would interfere with the ability of folate to target the complex. Nothing in Sinkule provides any indication that anything but the antibody targets the complex. Sinkule is not relying on folate targeting and provides no teaching or suggestion that folate could target such a complex.

The Office also states (page 6 of the August 4, 2010 Office Action):

> The instant claims are not drawn to the method of folate targeting of the folate-containing complex.

Claim 18, as summarized above, is directed to a method of targeting a radionuclide to a malignant cell within a subject, where the malignant cell expresses a tumor associated antigen and expresses folate binding protein. The method involves the use of a dual binding conjugate, where both the folate and the antibody components can target the malignant cell. As such, for the conjugate to have dual binding properties, the claimed invention requires the folate to maintain its targeting ability.

With regard to the folate component of the complexes encompassed by the present claims, the Office states (page 6 of the August 4, 2010 Office Action):

> The instant claims recite, "non-cytotoxic folate to form a dual binding conjugate." The folic acid analogues (chemotherapeutic agents) of the disclosures encompass the non-cytotoxic folate of the instant claims and therefore have the same properties and are capable of the same functions, such as forming dual binding conjugates. Further, the instant claims do not exclude that the non-cytotoxic folate is a chemotherapeutic agent.

Appellants submit that these statements are contrary to what a skilled artisan would consider to be a "chemotherapeutic agent" and are contrary to how chemotherapeutic agents are defined in Sinkule itself. Sinkule states (column 2, lines 40-45; emphasis added):

> By the term chemotherapeutic agent is meant a low molecular weight, i.e., less than 10,000 MW, chemotherapeutic agent *clinically useful against solid tumors, leukemias, viral infections or a variety of malignancies and pathological states.*

Clearly, in accordance with definition of a chemotherapeutic agent in Sinkule, the folic acid analogs that may be included in the conjugates described in Sinkule must be clinically useful against malignancies and pathological states. A *non-cytotoxic* folate, as recited in the present claims, does not meet this definition; by being non-cytotoxic, it is not "clinically useful against solid tumors, leukemias," etc. The disclosure of folates in Sinkule, therefore, does not encompass the *non-cytotoxic* folates recited in the present claims.

The other cited reference, Wedeking, describes targeting of small molecules using a folate (see columns 27-32). Even when larger complexes of multiple chelating agents are used and several folates are required in Wedeking (e.g., columns 51 and 52) the molecular weight is fairly low (less than 5000 for the molecule at columns 51 and 52, adding around 450 with three gadoliniums complexed). The Office does not appear to distinguish an antibody from a small molecule moiety. Appellants note, as stated above that the molecular weight of an IgG antibody is the order of 150,000 amu (atomic mass units). As such, an IgG antibody is thirty times larger than the largest complexes of Wedeking and three orders of magnitude larger than a folate. Given the vast size differences between a folate and an antibody, folate targeting and antibody targeting cannot simply be treated as if they are equivalent. Nothing in the combination of Wedeking with Sinkule teaches or suggests that the vanishingly small folate moiety (relative to an antibody) can have any useful positive effect in altering the distribution of

a massive antibody or, conversely, that including an antibody in a complex, such as that of Wedeking, that is targeted using a folate does not interfere with targeting by that folate.

For the above reasons, the combination of Sinkule with Wedeking fails to render a dual binding conjugate containing a radionuclide, an antibody, and a non-cytotoxic folate obvious. The obviousness rejection of claim 18 and its dependent claims should be withdrawn.

Claim 31

Claim 31 is directed to a conjugate consisting of (i) a radionuclide, (ii) an antibody, antibody fragment, or antibody construct, with affinity for a tumor associated antigen, and (iii) at least one non-cytotoxic folate. The inclusion of a "non-cytotoxic" folate in the conjugate is useful a targeting moiety, but, by virtue of being non-cytotoxic, is not a therapeutic for use in conjunction with the radionuclide. As indicated by Sinkule, certain folate analogues can be used as chemotherapeutics, but there simply is no teaching or suggestion in the cited art to include a "non-cytotoxic" folate in a complex containing an antibody component and a radionuclide.

As indicated above, while folate may be used to target a radionuclide, in view of Shinoda, the skilled artisan would have expected the antibody component in a complex to interfere with the ability of folate to bind its target. Inclusion of a "non-cytotoxic" folate would, therefore, not have been expected to be of any benefit and one skilled in the art would not have been motivated to include such a folate in an antibody/radionuclide complex. Appellants submit that claim 31 and its dependent claims are also free of the obviousness rejection over the combination of Sinkule and Wedeking.

Conclusion

Appellants respectfully request that the rejection of claims 18 and 25-35 be reversed. Appellants authorize the Office to charge $270.00 to Deposit Account No. 03-2095 in payment of the fee required by 37 C.F.R. § 41.20(b)(2). If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 6 September 2011

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Claims Appendix

18. A method of targeting a radionuclide to a malignant cell within a subject, wherein said malignant cell expresses a tumor associated antigen and expresses folate binding protein, said method comprising (i) coupling an antibody, antibody fragment, or antibody construct having affinity for said tumor associated antigen to at least one non-cytotoxic folate to form a dual binding conjugate, (ii) coupling said radionuclide to said dual binding conjugate, and (iii) administering said radionuclide coupled to said dual binding conjugate to said subject.

25. The method of claim 18 wherein said antibody is a human antibody.

26. The method of claim 18 wherein said antibody is a human IgG or IgM monoclonal antibody.

27. The method of claim 18 wherein said dual binding conjugate comprises multiple non-cytotoxic folates.

28. The method of claim 18 wherein said non-cytotoxic folate is folic acid.

29. The method of claim 18 wherein the step of coupling said antibody to said at least one non-cytotoxic folate is carried out by means of an activated ester.

30. The method of claim 18 wherein said malignant cell is derived from brain, cervical, ovarian, or breast tissue.

31. A conjugate consisting of (i) a radionuclide, (ii) an antibody, antibody fragment, or antibody construct, with affinity for a tumor associated antigen, and (iii) at

least one non-cytotoxic folate.

32. The conjugate of claim 31 wherein said antibody is a human antibody.

33. The conjugate of claim 31 wherein said antibody is a human IgG or IgM monoclonal antibody.

34. The conjugate of claim 31 wherein said conjugate comprises multiple non-cytotoxic folates.

35. The conjugate of claim 31 wherein said non-cytotoxic folate is folic acid.

Evidence Appendix

Shinoda et al. (Journal of Pharmaceutical Sciences 87:1521-1526, 1998) submitted with an Information Disclosure Statement received by the U.S.P.T.O. on January 28, 2004 and considered by the Examiner on June 19, 2006.

In Vivo Fate of Folate–BSA in Non-Tumor- and Tumor-Bearing Mice

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Abstract □ KB tumor cells exhibit an increased number of folate receptors on their membrane. This receptor has been proposed as a promising target for tumor drug targeting. Therefore, the disposition of folate-conjugated bovine serum albumin (folate–BSA) was examined as a model system for drug targeting. Nude mice which had received KB tumor cell transplants were given bolus intravenous administration of either ^{111}In-labeled folate–BSA (^{111}In-folate–BSA; 1 mg/kg) or unmodified ^{111}In-BSA (^{111}In-BSA; 1 mg/kg). The disposition characteristics and pharmacokinetics of ^{111}In-folate–BSA were compared with those of the ^{111}In-BSA as a control. The half-life of the β-phase of ^{111}In-folate–BSA in plasma was 140 min. The tumor uptake rate index for ^{111}In-folate–BSA was 0.46 μL/min/g, and that for ^{111}In-BSA was 0.32 μL/min/g. This index of ^{111}In-folate–BSA was slightly higher than that of ^{111}In-BSA in vivo, by a factor of 1.4. In vivo experiments showed folate–BSA has a relatively long plasma duration. ^{111}In-folate–BSA also showed selective distribution to tumors, but not as great as recent results from in vitro experiments. Therefore, the low vascular permeability of BSA into solid tumor tissue and inhibition of folate-mediated ^{111}In-folate–BSA uptake by tumor cells from the blood may be the rate-limiting factor of distribution.

Introduction

Further progress in chemotherapy now depends on the optimization of drug delivery. Interest has recently been focused on the tumor-specific targeting of highly potent antitumor drugs which may have otherwise undesirable side effects caused by nonspecific distribution. Over the past several years, a considerable number of studies of tumor-specific carrier-conjugated macromolecules (active carriers) have been reported, including transferrin-conjugated,[1] fibroblast growth factor-conjugated,[2] muramyl dipeptide-conjugated,[3] and Arg-Gly-Asp-Ser (RGDS) sequence peptide-conjugated[4] drugs. Active carrier-conjugated nanospheres have also been investigated, including surface-modified[5] and biotinylated-liposomes.[6]

In his studies on tumor targeting, Leamon and co-worker focused on the folate receptor, for the following reasons.[7,8] First, a macromolecule conjugated to folate can, in theory, undergo receptor-mediated endocytosis into the cytoplasm of cells via the folate receptor.[9] However, few reports of such successful uptake of macromolecules into the cytoplasm by receptor-mediated endocytosis have been studied. Second, the number of folate receptors on the tumor cell surface is significantly increased.[10,11] Recent findings suggest that folate-binding protein, a folate receptor, is a cell surface marker for a number of tumor cells, and this increase in the folate receptor may be useful for tumor targeting. Third, internalization of folate-conjugated macromolecules is thought to occur via a nonlysosomal pathway.[12,13] Folate-conjugated macromolecules were not measurably degraded following internalization into cells,[13] and many studies on antisense DNA or RNA delivery using this nonlysosomal pathway have now been reported.[14] For example, Lee and co-workers investigated folate-conjugated polylysine-liposome as DNA delivery vehicles in vitro in an attempt to enhance the uptake of DNA to tumor cells.[15] This is because DNA must be packaged into a vehicle to avoid the lysosomal pathway.

In the present study, in vitro specific uptake of folate-conjugated bovine serum albumin (folate–BSA) to tumor cells as an active targeting factor and plasma duration of folate–BSA in non-tumor-bearing mice as a passive targeting factor were investigated. BSA is used to increase plasma duration of the molecule. This occurs because BSA is not captured by macrophages in the liver nor excreted by normal kidney excretion, thereby increasing the chance of contact between the folate–BSA and the folate receptor.

After confirming active targeting and passive targeting, the in vivo disposition characteristics and pharmacokinetics of folate–BSA as a model system in tumor-bearing mice were investigated to test the suitability of folate–BSA as a targeting molecule. In the future, it may be possible to exchange BSA for other antitumor macromolecular drugs. Additionally, we could conjugate other low-molecular weight antitumor drugs to the BSA molecule to enhance delivery.

Experimental Section

Materials and Animals—BSA was purchased from Sigma (St. Louis, MO), folic acid from Wako (Japan), minimum essential medium (MEM) from Nissui Pharmaceuticals (Japan), KB tumor cells from Dainippon Pharmaceuticals (Japan), and gel for filtration from Pharmacia fine chemical (Sephadex G25 and G75; Sweden). Other chemicals were of reagent grade and obtained commercially. Male ddY mice (22–25 g) were chosen as non-tumor-bearing mice, and male BALB/c-nu nude mice (14–17 g) were chosen as tumor-bearing mice. They were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan) and maintained under standard housing conditions. Water and a laboratory diet were provided ad libitum.

Preparation of Folate–BSA—Synthesis of folate–BSA was carried out.[7] Briefly, 37.5 mg of folic acid was dissolved in 4 mL of dimethyl sulfoxide. 1-Ethyl-3-(3-(diethylamino)propyl)carbidiimide (16.2 mg, Dojindo, Japan) was then added, and the mixture was stirred at 25 °C for 30 min. BSA (15 mg/mL) was dissolved in carbonate buffer (pH 9.0, 0.1 M). The BSA solution was added dropwise to the folic acid solution and stirred at 25 °C for 3 h, and the pH was adjusted to 9.0 with 0.1 N HCl. The reaction was stopped by the addition of ethanolamine (0.3 mL). The crude sample was purified by gel filtration. The concentration of folate–

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BSA and the molar ratio of folate to BSA were determined by UV spectrophotometry (U3210, Hitachi, Japan).

Folate–BSA and BSA were also labeled with fluorecein isothiocyanate (FITC) to investigate uptake of folate–BSA to KB tumor cells according to the method by Leamon.[7] Folate–BSA was radiolabeled with ^{111}In using the bifunctional chelating agent diethylenetriaminepentaacetic acid anhydride (Dojindo, Japan) to investigate the disposition of folate–BSA in vivo.[16]

Disposition of ^{111}In–Folate–BSA in Mice—KB tumor cells were maintained in a tissue culture flask with MEM by weekly transfer of 10^6 cells to fresh medium. KB tumor cells (2×10^7 cells) suspended in 0.4 mL of saline were inoculated into the dorsal subcutaneous tissue of mice. At 14 days after inoculation, tumor-bearing mice (21–25 g, tumor weight: 0.1–0.9 g) received a 1-mg/kg dose of ^{111}In-folate–BSA or ^{111}In-BSA by injection into the tail vein and were then housed in metabolic cages for urine collection. At specific times (1, 2, 5, 10, 15, 30, 60, and 180 min) after dosing, blood was collected from the vena cava under ether anesthesia. The animal was then sacrificed, and the kidney, spleen, liver, lung, heart, muscle, and tumor tissues were excised, rinsed with saline, weighed, and subjected to scintillation counting. ^{111}In-radioactivities were counted in a well-type NaI scintillation counter (ARC-500, Aloka, Japan).

Separately, the same experiments were conducted in non-tumor-bearing mice. ^{111}In-folate–BSA (1 mg/kg) was administered via the tail vein.

Pharmacokinetic Data Analysis—Plasma concentration–time data were analyzed by a two-compartment open model. Each pharmacokinetic parameter was determined using the nonlinear least-squares program MULTI.[17] Tissue distribution data were evaluated using a tissue uptake rate index calculated from each tissue clearance. The change in the amount of radioactivity in a tissue with time can be described as follows:

$$dT(t)/dt = (CL_{in} \times C(t)) - (K_{out} \times T(t)) \quad (1)$$

where $T(t)$ (% of dose/g) is the amount of radioactivity in 1 g of the tissue, $C(t)$ (% of dose/mL) is the plasma concentration of radioactivity, CL_{in} (μL/min/g) is the tissue uptake rate index from the plasma to tissue, and K_{out} (1/min) is the rate constant for efflux from the tissue.

In the present study, the efflux process could be considered to be negligible during the initial phase of the experiment,[18] since ^{111}In radioactivity was mostly retained in the tissue even after degradation of compounds. Ignoring efflux, eq 1 integrates to:

$$CL_{in} = X(t_1)/\int_0^{t_1} C(t)dt = X(t_1)/AUC_{0\rightarrow t_1} \quad (2)$$

where t_1 (min) is the time of sampling after injection and X is the dose.

According to eq 2, the tissue uptake rate index was calculated using the amount of radioactivity in the tissue following the appropriate interval of time and AUC up to that same time point. CL_{org} can then be expressed as follows:

$$CL_{org} = CL_{in} \times W \quad (3)$$

where W (g) is the total weight of the organ. Urinary clearance (CL_{urine}) was calculated using the accumulated amount excreted in urine.

Total body clearance (CL_{total}) was calculated from AUC for infinite time (AUC_∞) by the following equation.

$$CL_{total} = Dose/AUC_\infty \quad (4)$$

Results and Discussion

Preparation of Folate–BSA and KB Tumor Cell Uptake of FITC–Folate–BSA in Vitro—Folate–BSA was synthesized at a molar ratio of folate to BSA of 5.7:1. KB cells treated with FITC-labeled folate–BSA (FITC–folate–BSA) at 37 °C were identified by epifluorescence microscopy, but cells treated with FITC-labeled BSA (FITC–BSA) were not labeled (data not shown). Furthermore, no fluorescence was detected in KB tumor cells following incubation with FITC–folate–BSA and a 10 fold excess concentration of free folate (data not shown), suggesting that this concentration of folate could inhibit the uptake of FITC–folate–BSA by KB tumor cells by competitive inhibition of the folate receptor. Furthermore, the amount of FITC–folate–BSA taken up into KB tumor cells decreased as free folate concentration increased, with an IC_{50} (the folate concentration at which 50% of FITC–folate–BSA uptaken by KB tumor cells was inhibited) value of about 10 nM. This inhibition of FITC–folate–BSA uptake to KB tumor cells by folate suggests that FITC–folate–BSA was specifically taken up via folate receptor, comparable with the results of Leamon.[7]

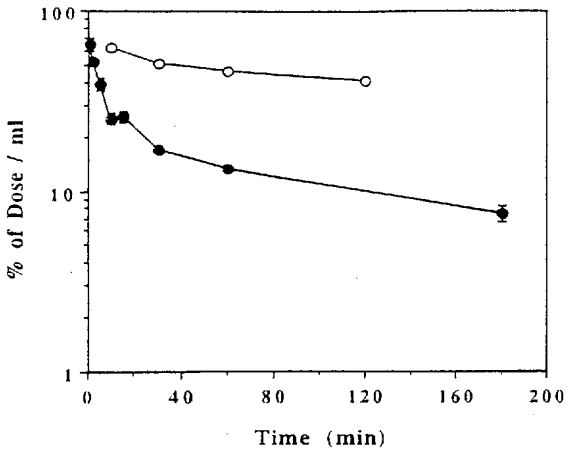

Figure 1—Plasma concentration of radioactivity in non-tumor-bearing mice after bolus intravenous administration of ^{111}In-folate–BSA and ^{111}In-BSA (1 mg/kg). ^{111}In–Folate–BSA (●); ^{111}In-BSA (○).

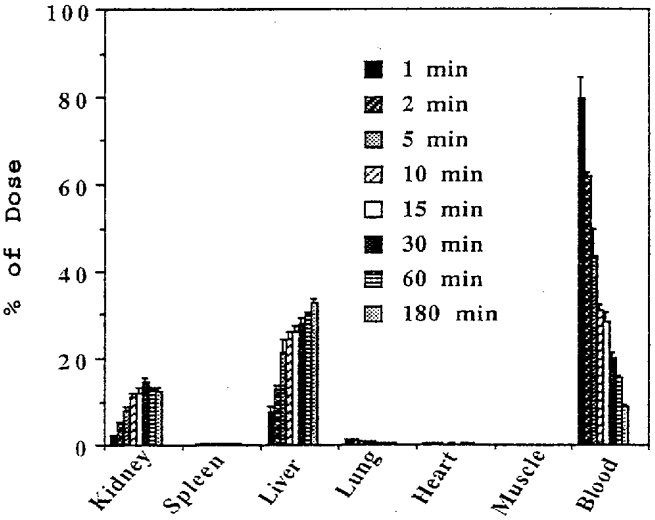

Figure 2—Tissue distribution of ^{111}In-folate–BSA in non-tumor-bearing mice after bolus intravenous administration (1 mg/kg). Each column and horizontal bar represent the mean ± SD of five mice.

Disposition of ^{111}In-Labeled Folate–BSA in Non-Tumor-Bearing Mice—To assess the duration time of ^{111}In-labeled folate–BSA (^{111}In-folate–BSA) in non-tumor-bearing mice plasma, plasma concentrations of ^{111}In-folate–BSA were investigated and compared with that of the other ^{111}In-labeled BSA conjugates [galactosylated-BSA (^{111}In-gal-BSA), succinylated-BSA (^{111}In-suc-BSA), cationized-BSA (^{111}In-cat-BSA), and unmodified BSA (^{111}In-BSA)] reported.[19–22] Male rats, which were different from tumor-bearing mice (nude mice), were used to compare folate–BSA in disposition to other BSA conjugates. On bolus

Table 1—Pharmacokinetic Parameters of ^{111}In-Folate–BSA in Non-Tumor and Tumor-Bearing Mice after Bolus Intravenous Administration (1 mg/kg)[a]

compound	MW	pI	number of "NH_2" group	$t_{1/2\,\alpha}$ (min)	$t_{1/2\,\beta}$ (min)	V_{d1}[c] (ml)	V_{dss}[d] (ml)	$AUC_{0-\infty}$ (% of dose·min/mL)	CL_{tot} (μL/min)
					Non-Tumor				
^{111}In-folate–BSA	69500	nd	54	3.6	116	1.4	4.2	3710	27
^{111}In-cat-BSA[b]	67000	9.0–9.4	90	3.3	50.4	4.1	16.8	–	352
^{111}In-suc-BSA[b]	71000	3.2–3.3	20	4.3	182	1.7	16.7	1090	163
^{111}In-gal-BSA[b]	68500	–	60	–	–	–	–	80.4	1243
^{111}In-BSA[b]	67000	4.7–4.9	60	76.9	2020	1.6	3.2	85800	1.2
					Tumor				
^{111}In-folate–BSA	69500	nd	54	8.9	140	1.4	2.1	9380	11
^{111}In-BSA	67000	4.7–4.9	60	13.3	392	1.5	1.5	35100	3

[a] Values were calculated by fitting the plasma concentration curve to a biexponential equation using the nonlinear least-squares program MULTI.[17] [b] Parameters of ^{111}In-cat-BSA, ^{111}In-suc-BSA, ^{111}In-gal-BSA and ^{111}In-BSA in non-tumor-bearing mice were reported previously.[19–22] [c] V_{d1} is distribution volume in central compartment (compartment 1). [d] V_{dss} is steady state $V_{d1} = V_{d2}$ distribution volume, $V_{dss} = V_{d1} + V_{d2}$.

Table 2—Tissue Distribution (% of dose) of ^{111}In-Folate–BSA in Non-Tumor-Bearing Mice after Bolus Intravenous Administration (1 mg/kg)

time (min)	kidney (%)	spleen (%)	liver (%)	lung (%)	heart (%)	muscle (%)	blood (%)
10	11.18	0.35	24.63	0.59	0.16	0.10	30.96
30	14.51	0.42	28.16	0.48	0.18	0.07	19.79
60	12.74	0.36	30.10	0.54	0.23	0.09	15.40
180	12.39	0.42	32.79	0.42	0.31	0.08	8.70

intravenous administration of ^{111}In-folate–BSA at 1 mg/kg to non-tumor-bearing mice, radioactivity disappeared from circulation in a biphasic pattern (Figure 1). The pharmacokinetic parameters of ^{111}In-folate–BSA mice were calculated according to a two-compartment model (Table 1). The half-life of the β-phase ($t_{1/2\,\beta}$) of ^{111}In-folate–BSA (116 min) was longer than that of ^{111}In-cat-BSA (50.4 min), and the AUC of ^{111}In-folate–BSA (3710% of dose·min/mL) was greater than that of ^{111}In-suc-BSA (1090% of dose·min/mL) and ^{111}In-gal-BSA (80.4% of dose·min/mL). The half-life of the α-phase ($t_{1/2\,\alpha}$) of ^{111}In-folate–BSA (3.6 min) was similar to that of ^{111}In-cat-BSA (3.3 min) and ^{111}In-suc-BSA (4.3 min; Figure 1, Table 1).[21] Thus, ^{111}In-folate–BSA had longer plasma duration and greater AUC compared to the other ^{111}In-BSA conjugates in non-tumor-bearing mice.

The disposition characteristics of ^{111}In-folate–BSA after bolus intravenous administration in non-tumor-bearing mice were investigated. The majority of the ^{111}In-folate–BSA was distributed to blood, liver, and kidney (Figure 2, Table 2). The total body, renal, and hepatic clearances of ^{111}In-folate–BSA were compared with values for the other ^{111}In-BSA conjugates.[20–22] The total body clearance (CL_{tot}) of ^{111}In-folate–BSA (27 μL/min) was smaller than that of ^{111}In-cat-BSA (352 μL/min), ^{111}In-suc-BSA (163 μL/min), and ^{111}In-gal-BSA (1243 μL/min; Table 1). The disposition of ^{111}In-folate–BSA was characterized by the same renal and urinary clearance (CL_{kidney}, CL_{urine}) as that of ^{111}In-gal-BSA, ^{111}In-suc-BSA, and ^{111}In-cat-BSA (2–10 μL/min), but with a very smaller hepatic clearance (CL_{liver}; 13 μL/min) than that of ^{111}In-gal-BSA (945 μL/min), ^{111}In-cat-BSA (248 μL/min), and ^{111}In-suc-BSA (136 μL/min; Table 3). The difference in plasma duration between ^{111}In-folate–BSA and the other ^{111}In-BSA conjugates was therefore due to a difference in hepatic clearance.

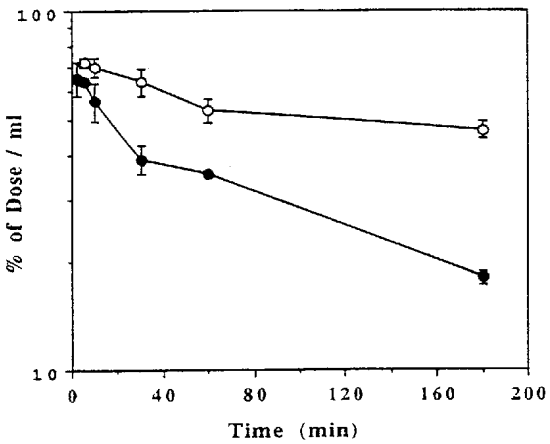

Figure 3—Plasma concentration of radioactivity in tumor-bearing mice after bolus intravenous administration of ^{111}In-folate–BSA and ^{111}In-BSA (1 mg/kg). ^{111}In–Folate–BSA (●); ^{111}In-BSA (○).

However, the $t_{1/2\,\beta}$ of ^{111}In-folate–BSA (116 min) was shorter than that of ^{111}In-BSA (2020 min), and the $t_{1/2\,\alpha}$ of ^{111}In-folate–BSA (3.6 min) was shorter than that of ^{111}In-BSA (76.9 min; Table 1). Therefore, ^{111}In-folate–BSA showed a relatively long plasma duration compared with other ^{111}In-BSA conjugates, but not as long as ^{111}In-BSA in non-tumor-bearing mice.

Generally speaking, efficient tumor targeting requires both "active targeting" and "passive targeting". Active targeting means increasing a molecule's affinity for the target site, while passive targeting means reducing nonspecific interaction with nontarget sites. Thus, the present study demonstrated that FITC–folate–BSA was specifically taken up into KB cells in vitro demonstrating active

Table 3—Clearances and Tissue Uptake Rate Indexes for ^{111}In-Folate–BSA in Non-Tumor-Bearing Mice after Bolus Intravenous Administration (1 mg/kg)[a]

compound	clearance (μL/min)				tissue uptake rate index (μL/min/g)				
	CL_{tot}	CL_{kidney}	CL_{liver}	CL_{urine}	kidney	spleen	liver	lung	heart
^{111}In-folate–BSA	27	4.9	13	2.4	15.3	1.5	7.6	0.9	1.2
^{111}In-cat-BSA[b]	352	7.3	248	2.7	23.0	148	217	74.3	5.5
^{111}In-suc-BSA[b]	163	7.1	136	–	21.5	2.4	95.8	2.3	1.7
^{111}In-gal-BSA[b]	1243	–	945	9.5	8.3	1.4	503	–	–
^{111}In-BSA[b]	1.2	0.5	0.4	–	1.1	0.4	0.2	0.7	0.4

[a] Organ clearance and tissue uptake rate values are calculated for ^{111}In-folate–BSA over 3 h, ^{111}In-cat-BSA over 2 h, ^{111}In-suc-BSA over 2 h, and ^{111}In-BSA over 2 h. [b] Values for ^{111}In-cat-BSA, ^{111}In-suc-BSA, ^{111}In-gal-BSA, and ^{111}In-BSA in non-tumor-bearing mice were reported previously.[19–22]

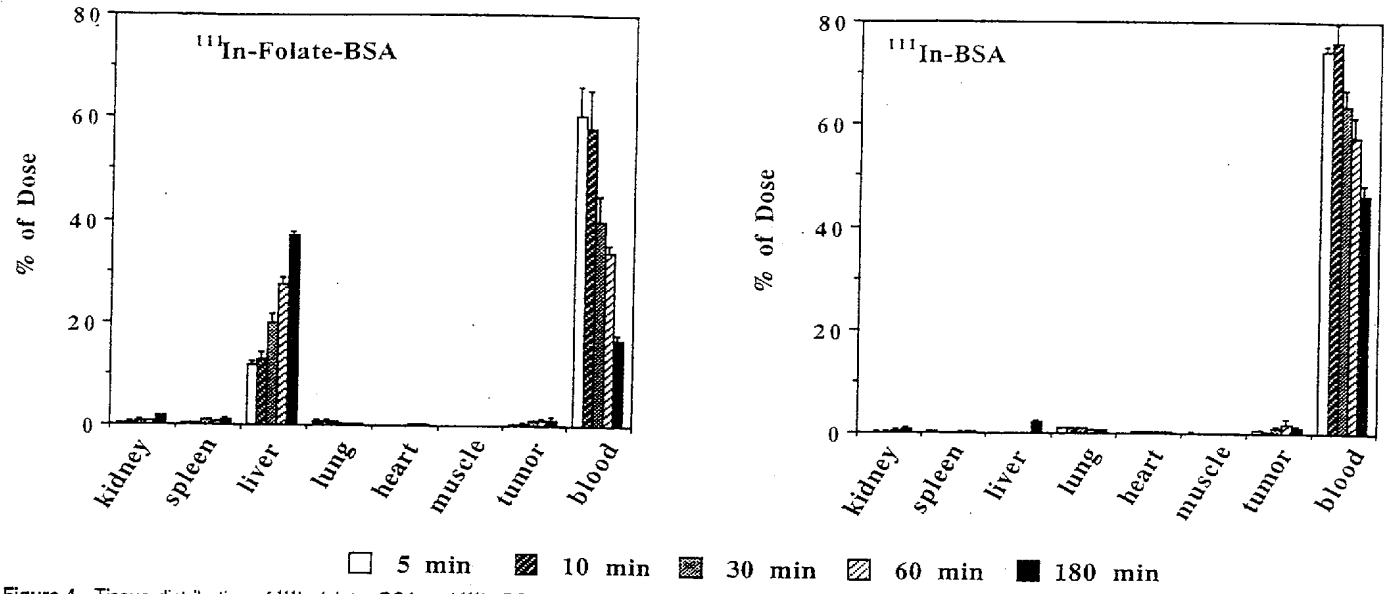

Figure 4—Tissue distribution of ^{111}In-folate–BSA and ^{111}In-BSA in tumor-bearing mice after bolus intravenous administration (1 mg/kg). Each column and horizontal bar represent the mean ± SD of five mice.

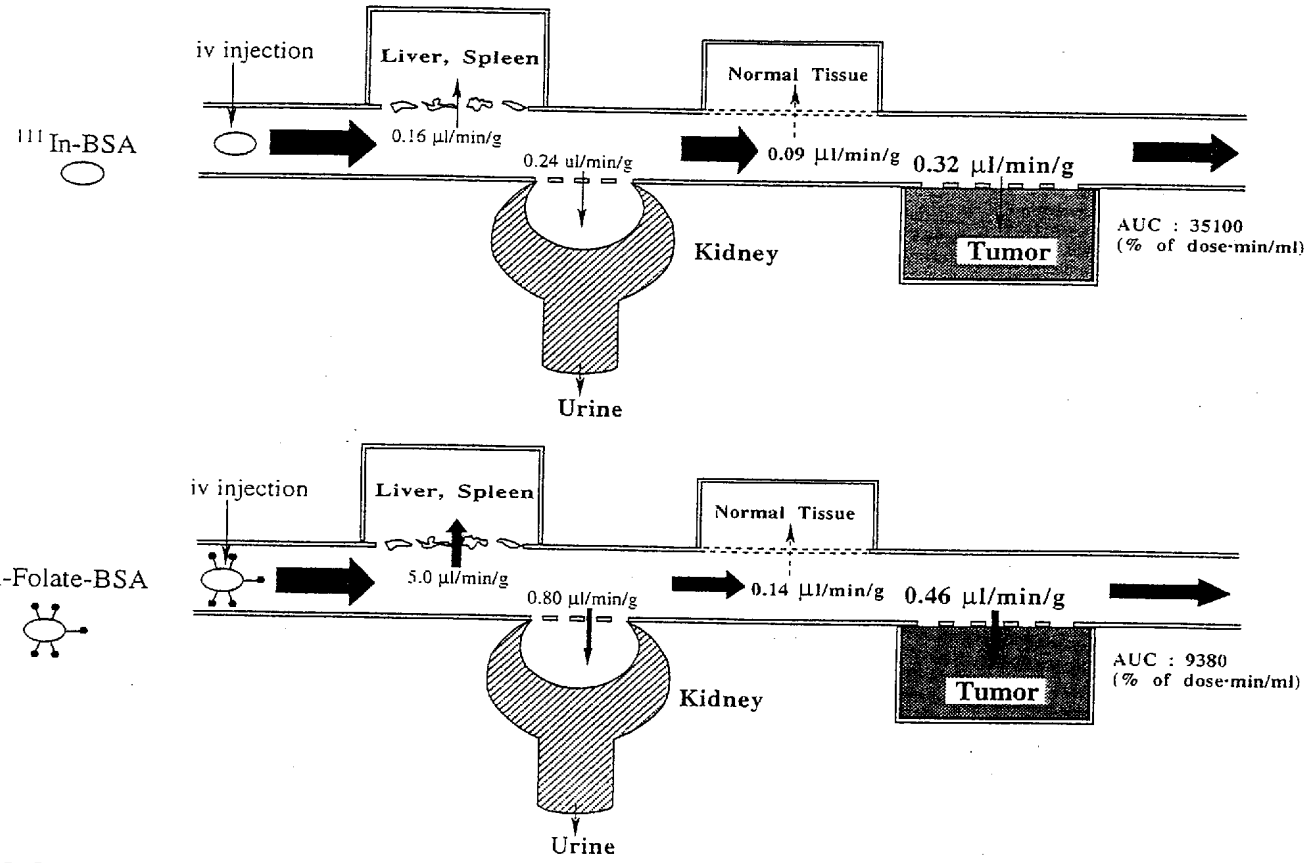

Figure 5—Summary of in vivo disposition characteristics of ^{111}In-folate–BSA and ^{111}In-BSA in tumor-bearing mice after bolus intravenous administration (1 mg/kg).

targeting and had a long plasma duration after bolus intravenous administration of ^{111}In-folate–BSA in vivo demonstrating passive targeting, indicating that ^{111}In-folate–BSA satisfies both requirements. This suggests that it may offer sufficient distribution to the target tissue (tumor) in tumor-bearing mice.

Disposition of ^{111}In-Folate–BSA in Tumor-Bearing Mice—To investigate the plasma duration time, the plasma concentration of ^{111}In-folate–BSA in KB cell tumor-bearing mice after bolus intravenous administration was measured. As in non-tumor-bearing mice, radioactive ^{111}In-folate–BSA in tumor-bearing mice disappeared from circulation

Table 4—Tissue Distribution (% of dose) of ^{111}In-Folate–BSA and ^{111}In-BSA in Tumor-Bearing Mice after Bolus Intravenous Administration (1 mg/kg)

time (min)	kidney (%)	spleen (%)	liver (%)	lung (%)	heart (%)	muscle (%)	tumor (%)	blood (%)
				^{111}In-folate–BSA				
10	0.20	0.34	12.67	0.75	0.11	0.04	0.48	58.09
30	0.84	0.92	19.92	0.45	0.15	0.03	0.90	40.00
60	0.81	0.67	27.37	0.45	0.20	0.04	1.10	33.79
180	1.61	1.05	36.97	0.47	0.39	0.05	1.22	16.62
				^{111}In-BSA				
10	0.35	0.10	0.55	0.66	0.12	0.03	0.32	75.50
30	0.50	0.20	0.55	0.65	0.11	0.01	0.70	65.32
60	0.82	0.10	0.22	0.55	0.13	0.02	1.08	58.91
180	1.20	0.21	3.96	0.35	0.12	0.01	1.07	48.51

in a biphasic pattern and had a relatively long plasma duration (Figure 3). The $t_{1/2\,\alpha}$ of ^{111}In-folate–BSA (8.9 min) was similar to that of ^{111}In-BSA (13.3 min), whereas $t_{1/2\,\beta}$ of ^{111}In-folate–BSA (140 min) was about half that of ^{111}In-BSA (392 min; Table 1).

To investigate ^{111}In-folate–BSA disposition, the tissue distribution of both ^{111}In-folate–BSA as a treatment and ^{111}In-BSA as a control in tumor-bearing mice was investigated. Tissue distribution of radioactivity for ^{111}In-folate–BSA showed its greatest accumulation in the liver tissue (12.67%) within 10 min. In contrast, ^{111}In-BSA did not accumulate in the liver tissue (0.55%; Figure 4, Table 4). The shorter $t_{1/2\,\beta}$ of ^{111}In-folate–BSA compared to that of ^{111}In-BSA could be due to hepatic clearance differences between ^{111}In-folate–BSA and ^{111}In-BSA. In tumor-bearing mice, ^{111}In-folate–BSA accumulation in the kidney tissue was 1.61%, but was 12.39% at 180 min in non-tumor-bearing mice (Figures 2 and 4, Tables 2 and 4). Total body clearance (CL_{tot}) of non-tumor-bearing mice (27 μL/min) was larger than that of tumor-bearing mice (11 μL/min; Table 1). This difference may be due to the difference in animals particularly with regard to different glomerular filtration between animals. The amount of ^{111}In-folate–BSA in other tissue such as spleen, lung, heart, and muscle tissues was very small.

A summary of in vivo disposition characteristics of ^{111}In-folate–BSA in tumor-bearing mice is shown in Figure 5. Pharmacokinetic analysis of the tissue distribution data demonstrated that the tumor uptake rate index for ^{111}In-folate–BSA (0.46 μL/min/g) was slightly higher, by a magnitude of 1.4, than that for ^{111}In-BSA (0.32 μL/min/g). With regard to macromolecule targeting, more than 80% of lactosyl-polystyrene (molecular weight: 60 kD) was targeted to hepatocytes after bolus intravenous administration,[23] indicating at the very least the possibility of macromolecule targeting to target sites. However, ^{111}In-folate–BSA could not be targeted to the tumor sufficiently in this experiment.

There are two reasons for this failure. First, this could be due to tissue structure differences between liver and tumor tissues. There are sinusoidal or discontinuous capillaries in the liver, resulting in gaps where the endothelial wall and basement membrane is absent or discontinuous. The endothelial cells in the sinusoids of the liver have so-called "sieve-plates", through which small particles (less than 100 nm) can pass. On the other hand, in tumors, the capillary is lined with tumor cells. These parts of contact can be either leaky, where the basement membrane is disrupted or absent, or others exhibit a continuous type of structure where there are formidable barriers to dispersion due to nonuniform blood supply or abnormally high pressure in the interstitial matrix.[24] The total recovery of ^{111}In-folate–BSA in tumor tissue at infinite time as calculated by the clearance parameter was smaller than that of ^{111}In-BSA, suggesting that the low vascular permeability into solid tumor tissue of the large ^{111}In-folate–BSA molecule may be the rate-limiting factor of accumulation.

Second, this failure could be due to inhibition of folate-mediated ^{111}In-folate–BSA uptake by KB tumor cells from the blood. In humans, the normal blood folic acid concentration is 3 nM. Assuming the blood concentration of folic acid is the same in mice and humans, 3 nM folate inhibited ^{111}In-folate–BSA uptake by KB tumor cells by only10%. Therefore, 90% of the folate–BSA was theoretically free to be taken up by KB tumor cells.

In conclusion, the FITC–folate–BSA synthesized in this study had high affinity for KB tumor cells in vitro. Further, the tumor uptake rate index of ^{111}In-folate–BSA was greater than that of ^{111}In-BSA in vivo, but not as great as recent results from in vitro experiments. These findings demonstrate that the targeting of drug–macromolecule conjugates to solid tumors must consider not only the affinity of the ligand (in this case, folate to the tumor cells) but also the in vivo fate of the carrier molecule and folic acid in blood.

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Related Proceedings Appendix

None.